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<b>(21) International Application Number:</b> PCT/US91/00559 <b>(22) International Filing Date:</b> 25 January 1991 (25.01.91) <b>(30) Priority data:</b> 470,067                      25 January 1990 (25.01.90)      US 591,559                      2 October 1990 (02.10.90)        US <b>(71) Applicant:</b> CHILDREN'S HOSPITAL [US/US]; 320 Longwood Avenue, Boston, MA 02115 (US). <b>(72) Inventors:</b> MOSES, Marsha, A. ; 80 Ivy Street, Brookline, MA 02146 (US). SUDHALTER, Judith ; 416 Grove Street, Apartment C-1, Newton Lower Falls, MA 02162 (US). <b>(74) Agents:</b> PABST, Patrea, L. et al.; Kilpatrick & Cody, 100 Peachtree Street, Suite 3100, Atlanta, GA 30303 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), NL (European patent), SE (European pa- tent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD AND COMPOSITIONS FOR INHIBITING ANGIOGENESIS  <b>(57) Abstract</b>  Pharmaceutical compositions for delivering an effective dose of an angiogenesis inhibitor consisting of a specific inhibitor of collagenase, for inhibition of blood vessel ingrowth and tube formation <i>in vivo</i> and capillary endothelial cell proliferation and migration <i>in vitro</i> , which is non-cytotoxic to normal cells. The compositions are delivered topically or locally using implants or injection. Examples of specific collagenase inhibitors include purified cartilage-derived inhibitor, collagenase binding peptides and antibodies which block the active site or metal binding regions of collagenase, and compounds which inhibit enzymes activating collagenase, such as plasmin inhibitor.		

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## METHOD AND COMPOSITIONS FOR INHIBITING ANGIOGENESIS

## Background of the Invention

5           The United States government has rights in this invention by virtue of grants from the National Institutes of Health.

          Angiogenesis, the process of new capillary formation, plays an important role in numerous  
10           physiological events, both normal and pathological. Angiogenesis is a complex process characterized by infiltration of basement membrane by capillary endothelial cells, the migration of cells through matrix towards a stimulus, and subsequent cell  
15           proliferation, resulting in new capillary tube formation.

          A number of proteases have been implicated as key factors in angiogenesis, but no one enzyme has been demonstrated to be critical in all three  
20           components of angiogenesis: endothelial cell migration and proliferation and capillary tube ingrowth. See, for example, Mignatti, et al., Cell 47, 487-498 (1986) and Rifkin, et al., Acta. Biol. Med. Germ. 40, 1259-1263 (1981), who suggest that a  
25           number of enzymes in a proteolytic cascade, including plasminogen activator and collagenase, must be inhibited in order to inhibit angiogenesis. Plasminogen activator and collagenase interact via plasmin, which is cleaved from plasminogen by  
30           plasminogen activator, and cleaves the latent form of collagenase to yield the active form. As a result, no one enzyme inhibitor has been shown to be able to inhibit angiogenesis in the absence of cytotoxicity.

          Under normal conditions, angiogenesis is  
35           associated with such events as wound healing, corpus luteum formation and embryonic development, as discussed by Folkman, et al., Science 243, 1490-1493 (1989). However, a number of serious diseases are also dominated by abnormal neovascularization

including solid tumor growth and metastases, some types of eye disorders, and rheumatoid arthritis, reviewed by Auerbach, et al., J. Microvasc. Res. 29, 401-411 (1985); Folkman, Advances in Cancer Research, eds. Klein and Weinhouse, pp. 175-203 (Academic Press, New York 1985); Patz, Am. J. Ophthalmol. 94, 715-743 (1982); and Folkman, et al., Science 221, 719-725 (1983). For example, there are a number of eye diseases, many of which lead to blindness, in which ocular neovascularization occurs in response to the diseased state. These ocular disorders include diabetic retinopathy, neovascular glaucoma, inflammatory diseases and ocular tumors (e.g. retinoblastoma). There are a number of other eye diseases which are also associated with neovascularization, including retrolental fibroplasia, uveitis, approximately twenty eye diseases associated with choroidal neovascularization and approximately forty eye diseases which are associated with iris neovascularization.

The current treatment of these diseases is inadequate, especially once neovascularization has occurred, and blindness often results. Studies have suggested that vaso-inhibitory factors which are present in normal ocular tissue (cornea and vitreous) are lost in the diseased state. The potential therapeutic benefit that a naturally occurring inhibitor of angiogenesis might have in controlling diseases in which neovascularization plays a critical role has prompted a long term search for angiogenesis inhibitors. An inhibitor of angiogenesis could have an important therapeutic role in relieving the course of these disorders, as well as provide a valuable means of studying their etiology.

A number of investigators have reported that extracts of cartilage, one of the few avascular tissues in the body, can inhibit angiogenesis:

Eisentein, et al., Am. J. Pathol. 81, 1-9 (1987);  
Pauli, et al., J. Natl. Cancer Inst. 67, 55-74 (1981);  
Brem and Folkman, J. Exp. Med. 141, 427-439 (1975);  
Langer, et al., Science 193, 70-72 (1976); Langer, et  
5 al., Proc. Natl. Acad. Sci. USA 77, 431-435 (1980);  
and Lee and Langer, Science 221, 1185-1187 (1983).  
Langer, et al., showed that cartilage extracts  
containing a collagenase inhibitor retard tumor-  
induced and inflammatory-induced neovascularization in  
10 the cornea and conjunctiva, when delivered by either  
infusion or sustained release from a polymeric  
implant.

Other inhibitors of collagenase in vitro have  
not inhibited angiogenesis in in vivo testing,  
15 including  $\alpha_2$ -macroglobulin and tissue inhibitor  
metalloproteinase (TIMP). Angiogenesis has several  
key components, including proliferation of capillary  
endothelial cells, migration of endothelial cells, and  
the production of collagenase. To inhibit  
20 angiogenesis, a compound, alone or in combination with  
one or more other factors, must inhibit proliferation  
of capillary endothelial cells, migration of  
endothelial cells, and collagenase. At the dosages  
tested, TIMP inhibits capillary endothelial cell  
25 proliferation but not angiogenesis, and protamine  
inhibits blood vessel ingrowth but not proliferation  
of capillary endothelial cells. Protamine was  
cytotoxic at concentrations appearing to inhibit  
angiogenesis.

30 Extracts from several other tissue sources have  
also been shown to have anti-angiogenic activity, as  
reviewed by D'Amore, Prog. Clin. Biol. Res. 221, 269-  
283 (1986). Several molecules have been identified  
which inhibit different aspects of angiogenesis, such  
35 as cell proliferation or cell migration, although no  
single molecule capable of inhibiting angiogenesis in  
vivo has been identified in the prior art until

reported by applicants in Science 248, 1408-1410  
(1990), as described in U.S. Serial No. 07/027,364  
filed March 18, 1987, Langer, et al. This  
application, which is a continuation-in-part of  
5 earlier applications reporting on a partially purified  
protein extract isolated from cartilage having anti-  
angiogenic activity, discloses the purification of a  
single protein that inhibits collagenase, and is also  
thought to be an inhibitor of angiogenesis, tumor-  
10 induced vascularization, connective tissue breakdown,  
and tumor cell invasion, and therefore useful in the  
treatment of corneal ulcers and rheumatoid arthritis  
and psoriasis, based on the inhibition of collagenase.

It is an object of the present invention to  
15 provide pharmaceutical compositions, and method of use  
thereof, for the treatment of diseases involving  
abnormal angiogenesis.

It is a further object of the present invention  
to provide pharmaceutical compositions, and method of  
20 use thereof, for inhibition of capillary endothelial  
cell proliferation and migration.

It is a still further object of the present  
invention to provide pharmaceutical compositions, and  
method of use thereof, which inhibit capillary  
25 endothelial cell proliferation and migration, as well  
as collagenase, or activation of collagenase, at  
pharmaceutically useful dosages.

It is another object of the present invention to  
provide topical and controlled release pharmaceutical  
30 compositions, and methods of use thereof, for  
inhibition of angiogenesis.

### Summary of the Invention

Pharmaceutical compositions for delivering an  
effective dose to a desired site of an angiogenesis  
35 inhibitor consisting of a collagenase inhibitor which

is not cytotoxic at effective dosages, being highly specific to collagenase. These compositions are based on the discovery that inhibition of collagenase alone can inhibit angiogenesis, if a sufficient concentration of the inhibitor is provided, and the effective concentration is not cytotoxic to normal cells. An example of a useful inhibitor is the protein derived from cartilage or cells which differentiate into chondrocytes, or proteins homologous thereto, for inhibition of blood vessel growth in vivo and capillary endothelial cell proliferation and migration in vivo. The protein can be extracted from bovine scapular cartilage using 2 M NaCl, followed by a series of precipitation and column chromatography steps; or expressed from nucleotide sequences encoding the protein, isolated using probes based on the NH<sub>2</sub>-terminal protein sequence. Other inhibitors include pieces of collagenase which bind to the active site or the region required for utilization of divalent ions, blocking antibodies, and compounds which inhibit activation of the precursor collagenase to active collagenase, either directly or by inhibition of the enzymes required for activation of collagenase, such as plasminogen activator inhibitor and plasmin inhibitor. Examples of proteins to be inhibited include stromelysin, transin, neutral proteinases, and thermolysin. Collagenase can also be inhibited by altering the pH of the microenvironment of the enzyme since it is active only at neutral pH.

The effective dosage for inhibition of angiogenesis in vivo, defined as inhibition of capillary endothelial cell proliferation and migration and blood vessel ingrowth, is extrapolated from in vitro inhibition assays. Effective dosages in vitro range from 1 nM, for inhibition of capillary endothelial cell migration to 100 nM, for inhibition

of capillary endothelial cell proliferation. The preferred dosage in vivo is between 16 nM and 50 to 75 nM for cartilage-derived inhibitor. The effective dosage is dependent not only on the specific inhibitor, but also on the method and means of delivery, which can be localized or systemic. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant.

In vitro assays have been developed to screen for compounds effective as angiogenesis inhibitors in vivo. These assay represent a key event required for angiogenesis, so that inhibition of the assay is representative of inhibition of angiogenesis in vivo. Events that are tested include proteolytic degradation of extracellular matrix and/or basement membrane; proliferation of endothelial cells, migration of endothelial cells, and capillary tube formation.

#### Detailed Description of the Invention

It has now been discovered, in contrast to numerous reports in the literature, that all of the different important factors of angiogenesis: proliferation and migration of endothelial cells, tube formation and ingrowth of blood vessels, can be inhibited solely by specifically inhibiting collagenase. This is most surprising in view of the many different pathways and enzymes involved in angiogenesis. It was not expected to find a common element of enough significance to allow specific inhibition of angiogenesis in the absence of cytotoxicity.



In vitro Assays for determining effectiveness and effective concentration of angiogenesis inhibitors in vivo.

Assays are used to screen compounds for inhibition of collagenase activity, proteolytic degradation of extracellular matrix or basement membrane, migration of endothelial cells, and capillary tube formation to determine (1) potential effectiveness in vivo as inhibitors of angiogenesis and (2) the effective concentration. As discussed above, all of these elements of angiogenesis must be inhibited in vivo for a composition to be effective. It has been determined that collagenase inhibitors are effective in all of these assays, if provided in a suitable carrier and concentration. The assays are therefore first used to determine if the compound is a collagenase inhibitor, then if it is active in the other assays and at what dosage.

The essential elements of these assays include using endothelial cells as the assay target and stimulation of the endothelial cells with known angiogenesis factors to compare a putative inhibitor's effect in unstimulated, as compared to stimulated, endothelial cells. Endothelial cells that can be used include capillary endothelial cells and umbilical vein endothelial cells. Factors which can be used to stimulate the endothelial cells include acidic or basic fibroblast growth factor.

The following assays are based on assays described in the prior art that have been modified for the purpose of screening collagenase inhibitors for inhibition of angiogenesis in vivo.

**Assay for Inhibition of Collagenase Activity.**

A unit of collagenase activity is defined as that amount of protein required to inhibit one unit of corneal collagenase by 50%. One unit of collagenase produces 10% cleavage of collagen in 2.5 h at 37°C. The

collagenase activity is measured using an assay developed by Johnson-Wint, Anal. Biochem. 68, 70 (1980). Type I collagen is purified from rat tail tendon by solubilization in 0.5 M acetic acid and repeatedly precipitated with sodium phosphate, and then acetylated using [ $^{14}\text{C}$ ] acetic anhydride. The radiolabelled collagen is dissolved in 0.5 M acetic acid together with sufficient non-labelled purified Type I collagen to make a solution of 2 mg/ml with a specific activity of approximately 350,000 cpm/ml. The collagen solution is dialyzed against two changes of 0.15 M sodium phosphate buffer, pH 7.6, at 4°C. Ninety-six well tissue culture plates are placed at a 45° angle and aliquots of the dialyzed solution (25  $\mu\text{l}$ , 8,750 cpm) are added to the lower half of each well. Fibrils are allowed to form by incubating the plates for 1 h at 37°C. Plates are immersed in water for 1 h, then allowed to dry overnight.

Collagenase is obtained from the culture medium of explanted bovine corneas. Corneas are excised from bovine eyes and rinsed in clinidine solution and then Hanks' balanced salt solution containing 2% penicillin-streptomycin. They are then cut into 4 mm<sup>2</sup> pieces and placed in T150 tissue culture flasks containing Dulbecco's modified Eagle's medium, 5% fetal bovine serum, and 2% penicillin-streptomycin solution. 0.5 ml of cytochalasin B (1 mg/ml in dimethyl sulfoxide) is then added to 100 ml of medium. One flask contains approximately 10 corneas/50 ml of medium. The flasks are incubated at 35°C in an incubator with humidified 95% air, 5% CO<sub>2</sub>. The medium is collected 7 days after corneas are explanted and stored at 4°C. Fresh medium is added to the corneas and a second collection made at day 14. Collagenase-containing medium is stored under sterile conditions for up to two months at 4°C. Before use, latent collagenase present in the culture medium is activated

by trypsin treatment, by incubating the medium for seven min at 37°C with one-tenth volume of 1 mg/ml of trypsin. Following activation, one-tenth volume of soybean trypsin inhibitor (5 mg/ml) is added to quench the trypsin. The enzyme is then diluted with collagenase assay buffer (CAB) (0.05 M Tris-HCl, pH 7.6, containing 0.2 M NaCl, 0.001 M CaCl<sub>2</sub>, and 0.01% sodium azide, CAB) such that 100 µl collagenase solution produces cleavage of 10% (5 µg) collagen in 2.5 h at 37°C.

Samples to be tested for collagenase inhibitory activity are dissolved in, or dialyzed against, CAB. 100 µl samples and 100 µl of diluted enzyme solution are mixed together, added to collagen-containing wells, and incubated for 2.5 h at 37°C. The supernatants, containing soluble radioactively labelled degradation products, are counted in a Beckman Model LS 1800 scintillation spectrometer.

#### Assay for Inhibition of capillary endothelial cell proliferation.

Capillary EC proliferation in response to an angiogenic stimulus is a critical component of neovascularization, as discussed by Ausprunk and Folkman, J. Microvasc. Res. 14, 153-65 (1977). By utilizing the specific cells involved in angiogenesis, and stimulating them with known angiogenesis factors, in this case acidic fibroblast growth factor (aFGF), as reported by D'Amore and Klagsbrun, J. Cell Biol. 99, 1545-1549 (1984), it is possible to mimic the angiogenesis process in vitro. This type of assay is the assay of choice to demonstrate the stimulation of capillary EC proliferation by various angiogenic factors, as reviewed by Shing, et al., Science 223, 1296-1298 (1984).

The number of endothelial cells in culture can be quickly determined based on the colorimetric determination of cellular acid phosphatase activity,

described by Connolly, et al., J. Anal. biochem. 152, 136-140 (1986). Capillary endothelial cells are plated ( $2 \times 10^3$  /0.2 ml) onto gelatin-coated 96-well tissue culture dishes on day 1. On day 2, cells are  
5 refed with Dulbecco's modified Eagle's medium (Gibco) with 5% calf serum (Hyclone) (DMEM/5) and aFGF (2 ng/ml) (FGF Co.) and increasing concentrations of compound added. The compound is added in volumes that do not exceed 10% of the final volume. Wells  
10 containing phosphate buffered saline (PBS) (Gibco) alone and PBS + aFGF are included as controls. On day 5, media are removed and cells are washed with PBS and lysed in 100  $\mu$ l of buffer containing 0.1 M sodium acetate (pH 5.5), 0.1% Triton X-100<sup>TM</sup> and 100 mM p-  
15 nitrophenyl phosphate (Sigma 104 phosphatase substrate). After incubation for 2 hours at 37°C, the reaction is stopped with the addition of 10  $\mu$ l of 1 N NaOH. Color development is determined at 405 nm using a rapid microplate reader (Bio-Tek).

20 According to Connolly, et al., a linear relationship is obtained between acid phosphatase activity and endothelial cell number up to 10,000 cells/well. Standard curves for acid phosphatase activity are also generated from known cell numbers in  
25 order to verify that the enzyme levels reflect the actual EC numbers. Percent inhibition is determined by comparing the cell number of wells exposed to stimulus with those exposed to stimulus and inhibitor. Each point represents the mean of quadruplicate  
30 control wells and triplicate inhibitor wells and the SEM is less than 7% of the mean. Electronic cell counting assays confirm the accuracy of the data.

The effect of the compound on capillary EC synthesis of DNA, can also be determined by the  
35 suppression of the incorporation of radioactive thymidine by capillary EC in response to aFGF.

### Assay for Inhibition of capillary Endothelial Cell Migration.

A modification of the Boyden chamber technique, Falk, et al., J. Immunol. 118, 239-247 (1980), is used to study the effect of CDI on capillary EC migration. A blind-well Boyden chamber, as described in J. Exp. Med. 115, 453-456 (1962), consists of two wells (upper and lower) separated by a porous membrane. A known concentration of growth factor is placed in the lower wells and a predetermined number of cells and compound to be tested are placed in the upper wells. Cells attach to the upper surface of the membrane, migrate through and attach to the lower membrane surface. The membrane can then be fixed and stained for counting, using the method of Glaser, et al., in Nature 288, 483-484 (1980).

Migration is measured using blind well chambers (Neuroprobe, no. 025-187) and polycarbonate membranes with 8- $\mu$ m pores (Nucleopore) pre-coated with fibronectin (6.67  $\mu$ g/ml in PBS) (human, Cooper). Basic FGF (Takeda Co.) diluted in DMEM with 1% calf serum (DMEM/1) is added to the lower well at a concentration of 10 ng/ml. The upper wells receive  $5 \times 10^5$  capillary EC/ml and increasing concentrations of compound to be tested. Control wells receive DMEM/1, either with or without bFGF. The migration chambers are incubated at 37°C in 10% CO<sub>2</sub> for 4 hours. The cells on the upper surface of the membrane are then wiped off by drawing the membrane over a wiper blade (Neuroprobe). The cells which have migrated through the membrane onto the lower surface were fixed in 2% glutaraldehyde followed by methanol (4°C) and stained and hematoxylin. Migration is quantified by counting the number of cells on the lower surface in 16 oil immersion fields (OIF). Each point represents the mean  $\pm$  SEM of 4 wells. In control wells without

bFGF, the number of migrated capillary ECs is 61 +/- 7 cells.

Assay for Inhibition of Neovascularization in vitro.

5           The chick chorioallantoic membrane assay (CAM), described by Taylor and Folkman, Nature (London) 297, 307-312 (1982), is used to determine whether the compound is capable of inhibiting neovascularization in vivo. The effect of the compound on growing  
10       embryonic vessels is studied using chick embryos in which capillaries appear in the yolk sac at 48 h and grow rapidly over the next 6-8 days.

          On day 3 of development, fertilized chick embryos are removed from their shells and placed in  
15       plastic petri dishes (1005, Falcon). They are maintained in humidified 5% CO<sub>2</sub> at 37°C. On day 6, 4 µg samples of purified CDI are mixed in methylcellulose disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus.  
20       Following a 48 hour exposure of the CAMs to CDI, avascular zones, free of capillaries and small vessels are observed using a binocular dissecting microscope at x7<sup>-10</sup> magnification. The CAMs are injected intra-vascularly with India ink/Liposyn as described  
25       previously by Taylor and Folkman (1982).

          Tissue specimens are fixed in formalin at room temperature and rinsed in 0.1 M cacodylate buffer pH 7.4. The specimens are embedded in JB-4 plastic (Polysciences) at 4°C and 3 µm sections are cut using  
30       a Reichert 2050 microtome. Sections are stained with toluidine blue and micrographs taken on a Zeiss photomicroscope using Kodak TM x100 and a green filter.

**Assay for Inhibition of Capillary Tube Formation.**

Capillary tube formation is an important component of angiogenesis. An assay which is indicative of capillary tube formation activity consists of applying the compound to, or between, collagen gels and determining if capillary-like tubes are formed by cultured BCE cells. In control collagen gels, not treated with inhibitor, a monolayer of BCE cells appears as a network of branching, anastomosing cords of endothelial cells resembling capillary beds in vivo. In contrast, collagen gels treated with an inhibitor show major disruption of the BCE cells reorganization such that any organization into capillary-like tubes appears to be prevented.

**Examples of determination of in vivo anti-angiogenic activity of cartilage derived inhibitor based on in vitro assays.**

Purified protein derived from cartilage (CDI) was isolated and characterized as having at least three distinct activities important in inhibition of angiogenesis. On the basis of these activities, the protein can be delivered in a pharmaceutically acceptable carrier in a dosage inhibiting angiogenesis in vivo, by locally inhibiting capillary endothelial cell proliferation and migration and blood vessel ingrowth.

As used herein, CDI refers to any protein specifically inhibiting eukaryotic collagenase encoded by a nucleotide sequence hybridizing under standard conditions to the nucleotide sequence encoding the inhibitor protein isolated from cartilage, as described below, or from chondrocyte or fibroblast culture fluid. Because CDI is not species specific, it is possible to use CDI isolated from non-human sources to treat humans. CDI-like proteins that specifically inhibit collagenase are produced by a

number of different tissues. As a result, CDI proteins can also be expressed from genetically engineered sequences isolated by standard techniques from a library prepared from chondrocytes or other cells such as fibroblasts which can differentiate into cartilage, or other mammalian cells, using labeled synthetic oligonucleotides encoding portions of the CDI isolated from cartilage as probes. These probes can be synthesized using commercially available equipment or by a company that performs synthesis on a contract basis. Methods for screening libraries using labelled probes or antibodies to a purified protein are well known to those skilled in the art, as taught, for example, by Maniatis, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY 1989).

Partial purification and characterization of the collagenase inhibition activity of the protein are described in detail in U.S. Serial No. 07/027,364 filed March 18, 1987. The cartilage-derived inhibitor (CDI) is isolated from bovine cartilage via a 2 M NaCl extraction followed by precipitation with hydrochloric acid and two ammonium sulfate precipitations (25%, 60%). The inhibitor is applied to an A-1.5 m gel filtration column. Further purification is achieved by ion exchange chromatography, a second gel filtration step and reverse-phase high performance liquid chromatography.

CDI can be isolated from cartilage. Veal scapulae are obtained from a slaughterhouse within 24 h after slaughter. The half of the cartilage furthest from the bone is excised and scraped first with a periosteal elevator (Arista) and then with a scalpel blade (No. 10, Bard-Parker) until clean of connective tissue. Cartilage slices are stored at -20°C until needed.

Frozen cartilage is thawed and chopped to form pieces of 1/8 in<sup>3</sup> in a Cuisinart Model DLC 10.



Chopped slices (1 kg per batch) are extracted in 8 L. 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6, containing 2 M NaCl, 3 mM EDTA, and 0.02% sodium azide for 48 h at 4°C. The extract is filtered through a Nitex filter (153  $\mu$  mesh, Tetko), centrifuged at 12,500 x g for 1 h at 4°C and then filtered through both Nitex and multiple layers of cheesecloth. The filtered extract, approximately 7.4 L, is concentrated to 500 ml using a Pellicon Cassette System (Millipore) equipped with one PTCG cassette (molecular weight cutoff,  $M_r$  10,000). The concentrate is dialyzed against 10 mM Tris-HCl, pH 7.6 containing 0.15 NaCl and 0.02% sodium azide, and store at -20°C.

To acid precipitate contaminants of the inhibitor, 1 N HCl is added dropwise to the concentrated extract until the pH reaches 2.0. The extract is centrifuged at 25,000 x g for 30 min and the pellet discarded. 1 N NaOH is added dropwise to the supernatant until the pH is 7.6. The extract is centrifuged at 25,000 x g for 30 min and the pellet discarded to remove any acid insoluble contaminants from the inhibitor-containing solution.

$(\text{NH}_4)_2\text{SO}_4$  is added to the extract to 25% saturation and the precipitate discarded after centrifugation. More  $(\text{NH}_4)_2\text{SO}_4$  is added until 60% saturation is reached. the 60% saturation solution is centrifuged and the inhibitor-containing pellet stored at -20°C.

The thawed pellet is dissolved in 0.01 M Tris-HCl, pH 7.6 containing 4 M guanidine-HCl, 0.001 M  $\text{CaCl}_2$ , and 0.02% sodium azide, and applied to an A1.5m Sepharose<sup>TM</sup> size exclusion column (Pharmacia, Piscataway, NJ) (2.5 x 90 cm) at a flow rate of 30 ml/h, 4°C. Column effluents are monitored spectrophotometrically at 280 or 220 nm. The inhibitor elutes at an apparent molecular weight of 28,000 Da. All fractions containing inhibitor by

assay of collagenase activity are pooled and concentrated in an Amicon-stirred cell equipped with a YM10 membrane. The retentate is dialyzed against 0.05 M Tris-HCl, pH 7.6 containing 0.05 M  $\text{CaCl}_2$ , and 0.05% sodium azide, then applied to a Bio-Rex 70 <sup>TM</sup> cation exchange column (200-400 mesh, 2.6 x 7.5 cm) at a flow rate of 70 ml/h. The column is washed with ten column volumes of 0.05 M Tris-HCl, pH 7.6 containing 0.05 M  $\text{CaCl}_2$ , and 0.05% sodium azide to elute nonabsorbing protein. Bound material is eluted with a salt gradient, 0.05 to 0.75 M NaCl in 0.05 M Tris-HCl, pH 7.6, containing 0.001 M  $\text{CaCl}_2$  and 0.01% sodium azide. The inhibitor binds to the column in low salt, 0.05 M NaCl, and eluted up to about 0.2 M NaCl.

Fractions containing inhibitor are concentrated in the stirred cell and dialyzed against collagenase assay buffer, CAB, 0.05 M Tris-HCl, pH 7.6, containing 0.2 M NaCl, 0.001 M  $\text{CaCl}_2$ , and 0.01% sodium azide. The dialysate is applied to a Sephadex G-75 <sup>TM</sup> (superfine, Pharmacia) column (1.6 x 50 cm) column, equilibrated with CAB, at a flow rate of 10 ml/h. Fractions containing inhibitor eluted after the dominant peak of an  $A_{280}$  absorbing material, and were pooled and concentrated in the stirred cell to a volume of 1 ml. An equal volume of 0.05% trifluoroacetic acid in water is added to the sample. Reverse-phase high performance liquid chromatography is then conducted on a  $C_4$  column. A linear gradient of 0.05% trifluoroacetic acid in water to 0.05% trifluoroacetic acid in acetonitrile is used to elute bound protein. 100  $\mu$ l aliquots are removed and immediately dialyzed against CAB for assaying collagenase activity.

The collagenase inhibitor is purified to apparent homogeneity by reverse-phase high performance liquid chromatography. Total protein, total units,

specific activity recovery, and purification for each step are listed in Table I.

Table I: Purification of a bovine cartilage-derived collagenase inhibitor.

Step	Total protein (mg)	Total Units <sup>a</sup>	Specific activity (units/mg)	Recovery (%)	Purification (x fold)
concentrated extract	25,900	398,462	15.4		
acid and (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt.	1,520	84,444	55.6	21.2	3.6
Al.5m	192	42,667	222.2	10.7	14.4
Bio-Rex 70	28.5	25,000	877.2	6.3	57.0
Sephadex G75	4.5	12,712	2,824.9	3.2	183.4
RHPLC	0.15	3,000	20,000.0	0.75	1,298.5

a A unit is defined as that amount of protein required to inhibit one unit of corneal collagenase by 50%. One unit of collagenase produces 10% cleavage of collagen in 2.5 h at 37°C.

5 CDI-like protein is isolated from conditioned media (Serum free HAM's F12 media supplemented with ascorbic acid, Gibco) in which chondrocytes from bovine scapula have been cultured by exhaustively dialyzing the media against distilled water, then  
 10 applying the dialysate to a Sephadex G75 gel<sup>TM</sup> (superfine, Pharmacia) filtration column (1.6 x 50 cm) in 0.05 M Tris-HCl, pH 7.6, containing 0.2 M NaCl, 0.001 M CaCl<sub>2</sub>, and 0.01% sodium azide, at a flow rate of 10 ml/h. Fractions were collected and  
 15 characterized as a single band with a molecule weight of approximately 34,000 by SDS-PAGE under reducing conditions. These fractions inhibited collagenase, as measured above. The fractions were also active in

both proliferation and migration assays, as well as in the CAM angiogenesis inhibition assay, with 80% inhibition at 4 micrograms.

Automated Edman degradation of CDI was performed on an Applied Biosystems, Inc. Model 477A protein sequencer using the manufacturer's standard program NORMAL-1. The resulting phenylthio-hydantoin-amino acid fractions were subsequently identified using and on-line (ABI) Model 120A HPLC. A search of the NBRF-PIR protein sequence data base, National Biomedical Research Foundation, Division of Research Resources National Institute of Health, Bethesda, MD, ID# A20595 (1989), revealed that this protein differs from a collagenase inhibitor isolated from human amniotic fluid (which itself is virtually identical to that of a human skin fibroblast inhibitor with the exception of one residue difference), in only two amino acids, at position 17 (valine for leucine) and at position 27 (alanine for proline) for 28 N-terminal residues, reported by Docherty, et al., Nature 318, 66-69 (1985). Samples were not reduced and alkylated, therefore, (---) represent bonafide blanks which align by homology with expected cysteine residues of previously reported collagenase inhibitors; all three residues are in agreement, based on Docherty, et al., (1985); Carmichael, et al., Proc. Natl. Acad. Sci. USA 83, 2407-2411 (1986). Sequence differences between this collagenase inhibitor and others previously reported, for example by Murray, et al., J. Biol. Chem. 261, 4154-4159 (1986), may be ascribed to species variations and/or variable forms of this protein in the same tissue, although CDI appears as a single band of apparent Mr of 27,650 as determined by SDS-PAGE analysis followed by silver staining.

The N-terminal sequence of CDI is shown in Table II.

Table II. NH<sub>2</sub>-terminal protein sequence of cartilage-derived inhibitor.

	1	2	3	4	5	6	7	8	9	10
	---	Thr	---	Val	Pro	Pro	His	Pro	Gln	Thr
11	12	13	14	15	16	17	18	19	20	21
Ala	Phe	---	Asn	Ser	Asp	Val	Val	Ile	Arg	Ala
22	23	24	25	26	27	28				
Lys	Phe	[Val]	Gly	Thr	Ala	Glu				

Other collagenase inhibitors can also be used. It is possible to use a material as simple as a peptide complementary to and binding to the collagenase active site, or which inhibits utilization of divalent ions by the collagenase, an antibody which blocks the collagenase activity and compounds which inhibit activation of the precursor collagenase to active collagenase, either directly or by inhibition of the enzymes required for activation of collagenase. By virtue of their specificity, these compounds are non-cytotoxic. Other proteins include platelet factor 4 and tissue inhibitor metalloproteinase (TIMP), both of which are collagenase inhibitors and which might also contain a peptide sequence which has anti-collagenase and anti-angiogenic activity.

The following demonstrates the discovery of the additional activities characterizing the CDI, and the effective dosage of CDI to inhibit angiogenesis, as compared to inhibition of collagenase activity in vitro. The CDI is purified from cartilage as described above.

#### Example 1: Inhibition of Capillary Endothelial Cell Proliferation by CDI.

The first in vitro studies conducted with CDI focused on the effects of this factor on one of the important components of angiogenesis, capillary endothelial cell (BCE) proliferation.

A. Inhibition of Endothelial Cell  
Proliferation measured by colorimetric  
determination of cellular acid phosphatase  
activity and electronic cell counting.

5 The colorimetric determination of cellular acid  
phosphatase activity, described by Connolly, et al.,  
J. Anal. biochem. 152, 136-140 (1986), is used to  
quickly and sensitively screen small amounts of  
fractions from entire columns in the purification  
10 scheme for inhibition of capillary endothelial cell  
proliferation, as measured by color.

Screening each successive column step using this  
assay, it was determined that the two distinct  
biological activities, anti-collagenase and anti-  
15 proliferative activities, copurify throughout the  
purification scheme. The same results were also  
obtained using a second assay to study BCE  
proliferation based on electronic cell counting. In  
these studies, fractions enriched in collagenase  
20 inhibitory activity from each chromatographic step  
throughout the purification scheme were tested for  
ability to inhibit acidic fibroblast growth factor  
(aFGF) stimulation of BCE proliferation. CDI was  
found to inhibit this proliferation in a significant,  
25 dose-dependent manner. Purified CDI inhibits aFGF-  
stimulated capillary EC proliferation with an  $IC_{50}$   
(concentration of inhibitor at which 50% inhibition of  
proliferation is obtained) of 50 to 75 nM. A  
comparison of the  $IC_{50}$  values (the inhibitory  
30 concentration at which 50% inhibition of growth  
factor-induced stimulation is observed) of CDI from  
the four successive steps as determined by cell  
counting assays reflects an increase in potency of its  
anti-proliferative activity as the collagenase  
35 inhibitor is purified.  $IC_{50}$  values: A-1.5m activity:  
14  $\mu$ g/ml, Bio-Rex 70 activity: 9  $\mu$ g/ml, Sephadex G-

75 activity: 4  $\mu\text{g/ml}$ , Reverse-phase HPLC activity:  
0.8  $\mu\text{g/ml}$ .

**B. Inhibition of Endothelial Cell**

**Proliferation measured by DNA synthesis.**

5 In studies to determine the effect of CDI on  
capillary EC synthesis of DNA, CDI suppressed the  
incorporation of radioactive thymidine by capillary EC  
in response to aFGF in a dose responsive manner with  
an  $\text{IC}_{50}$  of approximately 300 nM.

10 **C. Comparison with CDI Inhibition of**  
**Endothelial Cell Proliferation with**  
**control.**

To examine the specificity of the inhibitor's  
antiproliferative effect, a number of other  
15 substances, of particular relevance as control  
factors, were tested for their ability to inhibit  
capillary EC proliferation. Other enzyme inhibitors  
such as trypsin ovoinhibitor, pancreatic trypsin  
inhibitor and  $\alpha_2$ -macroglobulin, a relatively non-  
20 specific collagenase inhibitor, as well as chondroitin  
sulfate A, a glycosaminoglycan found in cartilage, did  
not have a significant effect (less than 20%  
inhibition or stimulation, at all doses tested) on  
growth factor-stimulated capillary EC proliferation  
25 even when tested at concentrations of 50  $\mu\text{g/ml}$ . These  
factors also do not inhibit angiogenesis in vivo. In  
addition, although the inhibitor inhibits aortic  
endothelial cells (AE), with an effective  
concentration that is much greater ( $\text{IC}_{50}$  = 40  $\mu\text{g/ml}$ )  
30 than for capillary endothelial cells ( $\text{IC}_{50}$  = 14  
 $\mu\text{g/ml}$ ), the inhibitor has no detectable inhibitory  
effect on the growth of non-endothelial cell types,  
such as bovine aortic smooth muscle cells and Balb/c  
3T3 cells.

**Example 2: Inhibition of Capillary Endothelial Cell Migration by CDI.**

Another important component of the angiogenic process is the migration of capillary EC in response to an angiogenic stimulus, as reviewed by Ausprunk, et al., J. Microvasc. Res. 14, 53-65 (1977).

The inhibitor of EC migration copurified with the collagenase inhibitor throughout the purification. IC<sub>50</sub> values were 143, 38 and 8 µg/ml for CDI obtained at the A-1.5m, Bio-Rex 70 and Sephadex G-75 chromatography steps respectively. Purified CDI inhibits capillary EC migration with an IC<sub>50</sub> of 96 nM.

**Example 3: Inhibition in vivo of Neovascularization by CDI.**

The chick chorioallantoic membrane assay (CAM), described by Taylor and Folkman, Nature (London) 297, 307-312 (1982), was used as a third bioassay to determine whether CDI was capable of inhibiting neovascularization in vivo. The effect of CDI on growing embryonic vessels was studied using chick embryos in which capillaries appear in the yolk sac at 48 h and grow rapidly over the next 6-8 days.

On day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic petri dishes (1005, Falcon). They were maintained in humidified 5% CO<sub>2</sub> at 37°C. On day 6, 4 µg samples of purified CDI were mixed in methylcellulose disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus. Following a 48 hour exposure of the CAMs to CDI, avascular zones, free of capillaries and small vessels were observed using a binocular dissecting microscope at x7-10 magnification. The CAMs were injected intravascularly with India ink/Liposyn as described previously by Taylor and Folkman (1982).

The results show significant inhibition, as evidenced by a large avascular zone, caused by 4 µg of



purified CDI (14.5  $\mu$ M) applied locally in methylcellulose discs on the normally developing chorioallantoic membrane within 48 hours of exposure to inhibitor. In contrast, control CAMs implanted with empty methylcellulose disks never develop avascular zones. Histological studies of CDI-treated CAMs reveal a mesoderm which is thinner than normal and avascular relative to controls which show normal vascular development.

The centers of the avascular zones were completely free of India-ink filled capillaries; the centers of the zones contain the methylcellulose disks. This effect was observed in 33% of the eggs tested in at least two separate sets of CAM assays using several different batches of cartilage starting material. Histological sections of day 8 CAMs ( $\times$  800) reveal normal capillary development in untreated controls and controls containing an empty methylcellulose disk. In contrast, capillaries were absent in test CAMs treated with 4  $\mu$ g of CDI. The anti-angiogenic activity of CDI was monitored throughout its purification using this CAM assay and dose dilution curves were generated for the inhibitor from each successive purification step through homogeneity. Purified CDI causes 100% avascular zones in the CAM at 4  $\mu$ g; 25  $\mu$ g of CDI from the G-75 chromatography step causes 100% avascular zones on the CAM; 50  $\mu$ g of CDI from the Bio-Rex 70 column causes 80% avascular zones, and CDI from the A-1.5 m step shows no inhibition of vascularization on the CAM.

The cartilage-derived inhibitor is a potent inhibitor of angiogenesis since inhibition was observed with as little as 4  $\mu$ g. For comparison, the lowest reported doses for previously described angiogenesis inhibitors tested alone in the CAM assay are 50  $\mu$ g of protamine (which is also cytotoxic at this concentration), as reported by Taylor and Folkman

(1982), 200  $\mu$ g of bovine vitreous extract, as reported by Lutty, et al., Invest. Ophthalmol. Vis. Sci. 24, 53-56 (1983), and 10  $\mu$ g of platelet factor 4, as reported by Taylor and Folkman (1982). By comparison, the cartilage-derived inhibitor is an extremely potent inhibitor of angiogenesis in vivo. The lowest reported doses of angiogenesis inhibitors effective as combinations include heparin (50  $\mu$ g) and hydrocortisone (60  $\mu$ g), and B-cyclodextrin tetradecasulfate (14  $\mu$ g) and hydrocortisone (60  $\mu$ g), reported by Eisenstein, et al., Am. J. Pathol. 81, 1-9 (1987).

**Example 4: Inhibition of Capillary Tube Formation by CDI.**

Using the assay for directed migration of capillary endothelial cells from cultured BCE cells into collagen gels and determining if capillary-like tubes are formed by cultured BCE cells, CDI was demonstrated to be an effective inhibitor of angiogenesis. In control collagen gels, not treated with CDI, the monolayer of BCE cells appears as a network of branching, anastomosing cords of endothelial cells resembling capillary beds in vivo. In contrast, collagen gels treated with CDI show major disruption of the BCE cells reorganization such that any organization into capillary-like tubes appears to be prevented.

**Example 5: Inhibition of tumor-induced angiogenesis using the rabbit corneal pocket assay.**

CDI, purified from bovine scapular cartilage or conditioned media of scapular chondrocytes, was shown in the foregoing examples to inhibit growth-factor stimulated proliferation and migration of capillary endothelial cell proliferation in vitro, when tested at nanomolar concentrations. Since tumor growth is dependent on neovascularization, the CDI was tested for suppression of tumor-induced angiogenesis using

the rabbit corneal pocket assay. Implants of V<sub>2</sub> carcinoma were utilized to produce the neovascular response in the normally avascular cornea. Sustained release polymer pellets of ethylene of ethylene-vinyl acetate copolymer (Elvax) were impregnated with 40 µg each of the CDI and implanted between the tumor and the limbal edge of the cornea.

The tumors induced vessels to sprout four to six days after implantation. When mean maximal vessel lengths were measured, it was found that the CDI significantly inhibited capillary blood vessel growth in the treated versus untreated eyes (45 to 65% inhibition).

**Example 6: Preparation of Pharmaceutical Compositions containing CDI as the active ingredient.**

Examples 1 to 3 demonstrate that a single, tissue-derived macromolecule, CDI, is a potent inhibitor of angiogenesis. In vitro studies using this inhibitor indicate that it negatively modulates at least three key components of the angiogenic process, the proliferation and migration of capillary endothelial cells, and tube formation. The CAM studies indicate that it also inhibits angiogenesis in an in vivo model.

Similar results have been obtained from the chondrocyte-derived inhibitor, testing for inhibition of proliferation and migration of capillary endothelial cells. In comparisons of angiogenesis inhibition, the chondrocyte derived material showed more activity than the cartilage derived CDI, which is not glycosylated following isolation using the acid precipitation. The chondrocyte material is glycosylated. It is believed that materials that are naturally expressed and glycosylated have greater activity than those materials which have been deglycosylated or expressed from recombinant sequences. However, all can be delivered in an

appropriate pharmaceutical vehicle to pharmaceutical use.

Pharmaceutical compositions are prepared using CDI as the active agent to inhibit angiogenesis based on the specific application. Application is either topical or localized. For topical application, the purified CDI is combined with a carrier so that an effective dosage is delivered, based on the desired activity (i.e. ranging from an effective dosage, for example, of 14.5  $\mu\text{M}$  or 4  $\mu\text{g}$  to prevent localized angiogenesis and for inhibition of capillary endothelial cell migration to 100  $\mu\text{M}$  for inhibition of capillary endothelial cell proliferation. A topical CDI composition is applied to the skin for treatment of diseases such as psoriasis. The carrier may be in the form of an ointment, cream, gel, paste, foam, aerosol, suppository, pad or gelled stick.

A topical CDI composition for treatment of some of the eye disorders discussed above consists of an effective amount of CDI in an acceptable ophthalmic excipient such as buffered saline, mineral oil, vegetable oils such as corn or arachis oil, petroleum jelly, Miglyol 182, alcohol solutions, or liposomes or liposome-like products. Any of these compositions may also include preservatives, antioxidants, antibiotics, immunosuppressants, and other biologically or pharmaceutically effective agents which do not exert a detrimental effect on the CDI.

CDI compositions for local or regional administration, for example, into a tumor, will generally include an inert diluent. Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as

benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

For directed internal topical applications, for example for treatment of ulcers or hemorrhoids, or other lesions of mucosal membranes, the CDI composition may be in the form of tablets or capsules, which can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; or a glidant such as colloidal silicon dioxide. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

In a preferred form, the CDI is administered in combination with a biodegradable, biocompatible polymeric implant which releases the CDI over a controlled period of time at a selected site. Examples of preferred polymeric materials include polyanhydrides, polyorthoesters, polyglycolic acid, polylactic acid, polyethylene vinyl acetate, and copolymers and blends thereof. CDI can also be administered at a local site by infusion pump, for example, of the type used for delivering insulin or chemotherapy to specific organs or tumors.

We claim.

1. A composition inhibiting angiogenesis comprising an effective amount of a specific inhibitor of collagenase in a pharmaceutically acceptable carrier to inhibit angiogenesis at a selected site, in the absence of cytotoxicity to normal cells at the site.

2. The composition of claim 1 wherein inhibition of angiogenesis is measured as inhibition of capillary endothelial cell migration.

3. The composition of claim 1 wherein inhibition of angiogenesis is measured as inhibition of capillary tube formation.

4. The composition of claim 1 wherein inhibition of angiogenesis is measured as inhibition of endothelial cell proliferation.

5. The composition of claim 1 wherein the inhibitor is purified cartilage-derived inhibitor.

6. The composition of claim 5 wherein the inhibitor is extracted from cartilage.

7. The composition of claim 5 wherein the inhibitor is expressed from recombinantly engineered sequences derived from a mammalian cell library using probes or antibodies to cartilage-derived inhibitor.

8. The composition of claim 5 wherein an effective dosage produces a concentration at the site where angiogenesis is to be inhibited of between approximately 1 nM and 100 nM.

9. The composition of claim 1 wherein the collagenase inhibitor is in an acceptable ophthalmic carrier for topical application to the eye.

10. The composition of claim 1 wherein the collagenase inhibitor is in a pharmaceutically acceptable carrier for topical application to the skin.

11. The composition of claim 1 wherein the collagenase inhibitor is in a biodegradable, biocompatible polymeric delivery device.

12. The composition of claim 1 wherein the collagenase inhibitor is in a pharmaceutical carrier for topical application to mucosal membranes.

13. The composition of claim 1 wherein the collagenase inhibitor is a peptide binding to the active site or divalent metal ion binding site of collagenase.

14. The composition of claim 1 wherein the collagenase inhibitor is an antibody to collagenase which blocks the collagenase activity.

15. The composition of claim 1 wherein the collagenase inhibitor inhibits the activation of precursor collagenase into active collagenase.

16. The composition of claim 15 wherein the collagenase inhibitor is selected from the group consisting of plasminogen activator inhibitor and plasmin inhibitor.

17. A method for inhibiting angiogenesis comprising

administering an effective amount of a specific collagenase inhibitor in a pharmaceutically acceptable carrier to a site where inhibition of blood vessel ingrowth is desired to inhibit angiogenesis, in the absence of cytotoxicity to normal cells at the site.

18. The method of claim 17 wherein the amount is effective to inhibit capillary endothelial cell migration.

19. The method of claim 17 wherein the amount is effective to inhibit capillary endothelial cell proliferation.

20. The method of claim 17 wherein the amount is effective to inhibit capillary tube formation.

21. The method of claim 17 wherein the site is a tumor.

22. The method of claim 17 further comprising administering the collagenase inhibitor in a pharmaceutical vehicle suitable for topical application to the skin.

23. The method of claim 17 further comprising providing the collagenase inhibitor in a biodegradable, biocompatible polymeric delivery device.

24. The method of claim 17 further comprising providing the collagenase inhibitor in a pharmaceutical vehicle suitable for injection.

25. The method of claim 17 further comprising administering the collagenase inhibitor in a pharmaceutical vehicle suitable for topical application to the eye.

26. The method of claim 17 wherein the inhibitor is purified cartilage-derived inhibitor and an effective dose produces a concentration at the site where angiogenesis is to be inhibited in the range of between approximately 1 nM and 100 nM.

27. The method of claim 22 wherein the effective dose is a dose effective in diminishing the symptoms of psoriasis.

28. The method of claim 17 wherein the effective dose is a dose effective in diminishing the number of blood vessels growing into a tumor.

29. The method of claim 25 wherein the effective dose is a dose effective in diminishing the symptoms of eye diseases characterized by abnormal neovascularization.

30. The method of claim 17 wherein the collagenase inhibitor is a peptide binding to the active site or divalent metal ion binding site of collagenase.

31. The method of claim 17 wherein the collagenase inhibitor is an antibody to collagenase which blocks the collagenase activity.



32. The method of claim 17 wherein the collagenase inhibitor inhibits the activation of precursor collagenase into active collagenase.

33. A method for determining the efficacy of a compound for inhibition of angiogenesis in vivo comprising

determining the effective dose for inhibition of capillary endothelial cell migration,

determining the effective dose for inhibition of capillary endothelial cell proliferation, and

determining the effective dose for inhibiting blood vessel ingrowth and tube formation.

34. The method of claim 33 wherein the effect of the compound on endothelial cells is measured.

35. The method of claim 33 wherein the endothelial cells are selected from the group consisting of activated endothelial cells and unstimulated cells.

36. The method of claim 33 further comprising determining the effect of the compound on proteolytic degradation of extracellular matrix or basement membrane is determined.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/00559

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 37/00; 39/00; C07K 15/00; C12N 5/00 US. Cl.: 514/2; 424/85.8; 435/240.2						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; font-size: small;">Minimum Documentation Searched ?</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none;">US. Cl.</td> <td style="border: none;">514/2; 424/85.8; 435/240.2</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *</div> <p>Databases: Automated Patent Searching (1975-1991)          Dialog (Files: Medline, Biosis, CAS, World Patents Index)</p>			Classification System	Classification Symbols	US. Cl.	514/2; 424/85.8; 435/240.2
Classification System	Classification Symbols					
US. Cl.	514/2; 424/85.8; 435/240.2					
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> †						
Category *	Citation of Document, † with indication, where appropriate, of the relevant passages ‡	File and Publication No. §				
Y	Nature, Volume 318, issued 07 November 1985, Docherty et al, "Sequence of Human Tissue Inhibitor of Metalloproteinases and its Identity of Erythroid Potentiating Activity," pages 66-69, see page 66.	1-13+17-30				
Y	Biochimica et Biophysica Acta, Volume 924, issued 1987, Gabrielides et al., "A Vertebrate Interstitial Collagenase Inhibitor from Bovine Scapular Cartilage: Purification and Characterization", pages 238-247, see page 238.	1-13, 17-30 +33-36				
Y	Science, Volume 221, issued 19 August 1983, Folkman et al., "Angiogenesis Inhibition and Tumor Regression Caused by Heparin or a Heparin Fragment in the Presence of Cortisone", pages 719-725, see the Abstract.	21				
<div style="font-size: x-small;">           * Special categories of cited documents: †            "A" document defining the general state of the art which is not considered to be of particular relevance            "E" earlier document but published on or after the international filing date            "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            "O" document referring to an oral disclosure, use, exhibition or other means            "P" document published prior to the international filing date but later than the priority date claimed            "T" later document published after the international filing date, its priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention            "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step            "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.            "&amp;" document member of the same patent family         </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search  <div style="text-align: center; font-size: large;">22 April 1991</div>	Date of Mailing of this International Search Report  <div style="text-align: center; font-size: x-large; font-weight: bold;">24 MAY 1991</div>					
International Searching Authority  <div style="text-align: center; font-size: large;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;">           R. Keith Baker, Ph.D.  </div>					

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Science, Volume 221, issued 16 September 1983, Lee et al., "Shark Cartilage Contains Inhibitors of Tumor Angiogenesis", pages 1185-1187, see entire document.	1-13+17-30
Y	Journal of Immunological Methods, Volume 33, issued 1980, Falk et al., "A 48-Well Micro Chemotaxis Assembly for Rapid and Accurate Measurement of Leukocyte Migration" pages 239-247, see the Abstract.	33-36
Y	The Journal of Cell Biology, Volume 107, Number 6, Part 3, issued December 1988, Moses et al., "Bovine Scapalan Cartilage Contains an Inhibitor of Capillary Endothelial Cell Migration", page 374a, see Abstract 2132.	1-13, 17-30+ 33-36
Y	US, A, 4,766,111 (N. Catsimpoilas) 23 August, 1988, see the Abstract.	15, 16, 32
Y	US, A, 4,743,587 (Dickens et al.) 10 May 1988, see columns 1 and 5.	1-13+17-30
Y	Biological Abstracts, Volume 85, No. 11, issued 01 June 1988, Crespo et al., "Monoclonal Antibodies Against Synovial Collagenase: Use for Immunopurification and Characterization of the Latent and Active Enzyme", see page 115, column 1, abstract no. 107968, Collagen Relat. Res, 8(1), 1-10.	14 and 31